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13. ABSTRACT ***(Maximum 200 Words)***

We have transfected the 184B5 immortalized, non-transformed human mammary epithelial cell line with the oncogenic mutant *ras* gene and with empty vector. We have isolated the following stable transfecants: (1) cells overexpressing the mutant *ras* oncogene (2) cells overexpressing the normal, non-mutant form of *ras* (3) cells transfected with the empty vector. The first group of clones is being sorted by their ability to form tumors. We are currently performing cDNA microarray analysis qunatifying the expression level of about 15,000 genes in these cell lines. We have also performed cDNA micorarray analysis on several established breast cancer derived cell lines. The comparative analysis will reveal the gene expression similarity between the cell lines that were transformed by the ras oncogene. This will determine whether gene expression patterns could be used to infer that certain breast tumor samples were originated, at least partially, by the ras oncogene. We are also comparing the gene expression patterns derived from in vitro transformed cells to the gene expression profile of primary breast tumors and established breast cancer cell lines. This will help to assess the ratio of gene expression changes in primary breast tumors that could be attributed to the presence of the above listed oncogenic changes. If this ratio is low, then the potential role of other oncogenic factors in the development of breast cancer will be substantiated. The opposite result will support the definitive role of these already known oncogenes in sporadic breast cancer. We will also compare the tumor forming and the non-malignant clones that both overexpress the ras oncogenes and determine which ras inducible genes are relevant for tumor formation.

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Table of Contents

INTRODUCTION:

Despite major advances in the field, such as the discovery of BRCA1 and BRCA2, we have very limited information about the molecular cause of the majority of breast cancer cases. In fact, most of our current knowledge about sporadic breast cancer is based on the application of previously known cancer associated mechanisms for this type of tumor. Only a limited number of cancer related changes originated from the analysis of mammary carcinoma itself (such as BRCA1), which makes it rather probable that many breast cancer-associated or -specific genetic changes remain to be identified. There is a well-characterized group of oncogenic changes that is often present in breast cancer. These include the overexpression of the myc and erbB2 oncogenes and mutation of the ras gene. However, it is not known to what extent these genetic changes are responsible for the development and maintenance of sporadic breast cancer. For example, if breast cancer is mainly induced by genetic instability or aneuploidy then many of the known changes currently related to the overexpression of myc and erbB2 might be independent of these oncogenes and be simply a result of an overall rearrangement of gene expression pattern in cancer. If, however, these oncogenes play a strong causative role in sporadic breast cancer then the gene expression changes induced by the overexpression of these oncogenes must be also present in sporadic breast cancer samples as well.

The aim of this project is establishing an experimental system that will allow answering this question.

BODY:

During this intitial stage of the project we aimed to achieve the following goals:
a) Establishing breast epithelial cell lines that were transformed under identical in vitro conditions with the widely used oncogene, mutant ras.
b) Establishing the cDNA microarray technology on breast epithelial cells and start our microarray data library based on the gene expression patterns of breast cancer cell lines, normal breast epithelium and non-malignant, immortalized breast epithelium.
c) reaping the initial benefit of "guilt by association" analysis by identifying consistently mis-regulated genes in breast cancer.
The immediate goal of the project was to determine, how deterministic is the ras overexpression for gene expression patterns and how does that compare to gene expression patterns found in sporadic breast cancer.

a) 184B5 cells (1) , an immortalized cell line derived from normal human mammary epithelial cells were transfected with pUSE-ras(Q61L) (UpState Biotechnology), an eukaryotic expression vector containing the coding sequence of an activated form of the human H-ras gene. The activating mutation at codon 61 results in the substitution of leucine for glutamine, which in turn results in increased nucleotide exchange and reduced GTP hydrolysis, and thus maintenance of the activated (GTP-bound) state (2). As a negative control, cells were also transfected with empty pUSE vector (containing no cDNA insert). Stable transfecants were selected by geneticin resistance. Single cell colonies were selected with the aid of cloning rings and by dilution in 96-well plates. From the cells transfected with pUSE-ras(Q61L), 44 Geneticin-resistant clones were harvested, and from cells transfected with empty pUSE vector (negative control), 6 resistant clones were harvested. Probing of Western blots of the lysates with primary

antibodies against both ras and actin at the same time allowed ras expression levels to be normalized to actin expression. The luminescence intensities of the ras and actin bands for each sample were measured, and the ratio of the ras signal to the actin signal was calculated. Lysate from one of the empty vector clones, pUSE-5, was included on every Western blot as a reference. For each sample, a normalized ras:actin ratio was calculated, which was taken to be the ras:actin ratio of the sample divided by the ras:actin ratio of the pUSE-5 empty vector clone included on that blot. The mean ± standard deviation of the normalized ras:actin ratios of the 6 empty vector clones is 1.21 ± 0.22. Clones transfected with pUSE-ras(Q61L) which have a ras:actin ration at least 3 standard deviations above the mean ras:actin ration of the empty vector clones, which is 1.21 + (3)(0.22) = 1.87, were considered to be overexpressers of ras, and 14 of the 44 clones met this criteria, with normalized ras:actin ratios ranging from 1.98 to 14.1. Of the 14 overexpressing clones 11 showed good proliferative capacities.

We are currently testing the malignancy of the overexpressors by nude mouse tumor formation.

We also started the cDNA microarray analysis on these clones. For microarray analysis of a clone, 50 μg of its total RNA was used in a reverse transcription reaction containing Cy3-dUTP in order to generate Cy3-labeled cDNA, and this was hybridized to the microarray slide along with Cy5-labeled cDNA generated from 50 μg of total RNA from non-transfected 184B5 cells, which serves as the reference cell line for all microarray analyses. All microarray slides were printed and scanned at the National Human Genome Research Institute, National Institutes of Health, Bethesda, MD. The protocols for the cDNA synthesis reactions and the microarray hybridizations are available at www.nhgri.nih. gov/DIR/LCG/15K/HTML/.

At the completion of this report we are close to finishing the microarray studies in duplicates.

b) In addition to the 14 ras overexpressor cell lines, we have performed cDNA microarray analysis on the following breast cancer cell lines as well; BT-549, MCF-7, MDA-MB231, MDA-MB-157, MDA-MB-134, SK-BR-3, T47-D, ZR-75-1. This is our reference data base for further analysis and it has also provided several leads to identify consistently mis-regulated genes in breast cancer.

c) One of these consistently mis-regulated genes is the desmosomal cadherin, desmocollin 3. We have reported (3) that the down-regulation of this gene is frequently and consistently detected in sporadic primary breast cancer. We have re-transfected highly metastatic breast cancer cells with this gene and currently testing whether the reintroduction of this gene has reduced the malignancy of the parent cell line.

KEY RESEARCH ACCOMPLISHMENTS:

1) We have prepared 14 clones of Ras overexpressors of immortalized breast epithelial cells.

2) We have performed cDNA microarray analysis on the ras overexpressors and on breast cancer cell lines relative to non-malignant breast epithelium. The analysis of this large body of data is currently underway.

3) We have identified and reported Desmocollin 3 as a consistently down-regulated gene both in breast cancer cell lines and primary breast tumors.

REPORTABLE OUTCOMES:

1) 14 breast epithelial cell lines (originating from the 184B5 line) overexpressing the mutant ras oncogene available to all interested researchers.
2) cDNA microarray database on the gene expression patterns of the following breast cancer cell lines; BT-549, MCF-7, MDA-MB231, MDA-MB-157, MDA-MB-134, SK-BR-3, T47-D, ZR-75-1 and 14 ras overexpressor cell lines. Available to all interested researchers, soon to be posted on our website.

CONCLUSIONS:

We have created a set of clones overexpressing the mutant ras oncogene. All these clones were created under identical conditions allowed by the experimental technology. After determining which clone is oncogenic and which one is not we will compare this phenotypic information to the gene expression patterns that are currently being measured. This will allow us to determine whether malignant transformation induced under identical conditions will induce near identical gene expression patterns or not.

The limited nature and time frame of the concept award did not allow us to provide the final answer. This will be available within 2 months, after completing the tumor formation assays.

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Down-regulation of the desmosomal cadherin desmocollin 3 in human breast cancer

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Abstract. In previous studies using cDNA microarray analysis, we have identified an expressed sequence tag which is consistently down-regulated in six human breast tumor cell lines. In the current study, we have determined this tag to be part of the mRNA sequence of human desmocollin 3, a member of the cadherin superfamily of proteins and an integral component of desmosomes. Desmosomes are sites of adhesion between adjacent cells in layers of epithelia, as well as in some non-epithelial tissues, and play an important role in the maintenance of tissue structure. Northern analysis, quantitative real-time polymerase chain reaction assay and Western blot analysis showed that desmocollin 3 is present in normal and immortalized human mammary epithelial cells, but consistently exhibits a significant, and often complete, down-regulation in breast cancer cell lines and primary breast tumors, both at the mRNA and protein levels.

Introduction

The identification of gene expression changes associated with the development and maintenance of the tumor phenotype has been greatly facilitated recently by the development of technologies which enable large-scale measurement of gene expression patterns. One such technology is cDNA microarray analysis, in which cDNAs prepared from tumor cells and normal cells are labeled with different fluorescent dyes and applied to a glass slide imprinted with partial sequences from 5,000-10,000 genes (1). The ratio of fluorescence intensities from the dyes indicates whether a given gene is up-regulated, down-regulated or unchanged in a given tumor sample as compared to a normal control. In a microarray analysis comparison that we have recently performed between 6 human breast tumor cell lines and an immortalized non-malignant breast epithelial cell line, 12 cDNA sequences were found to have a reduced expression level in all 6 tumor cell lines (2). The probability of a given gene exhibiting reduced expression in all 6 tumors by chance (i.e., as a result of random variations in gene expression in the various tumors) is extremely low (Wahde *et al*, unpublished data) and, therefore, sequences identified by this method merit further study. Of the consistently down-regulated sequences, the one exhibiting the greatest degree of down-regulation is a partial-length cDNA from a human placenta cDNA library, which at the time of analysis had no similarity with any mRNA in GenBank. As reported in the current study, the subsequent isolation and sequencing of a longer clone containing this sequence revealed it to be part of the 3' UTR of human desmocollin 3. Desmocollin 3 (3,4) is a member of the cadherin superfamily of calcium-dependent cell adhesion molecules and a principal component of desmosomes, which are proteinaceous plaques that span the plasma membrane and serve as sites of adhesion between neighboring cells, primarily in layers of epithelia, but also in cardiac muscle, the follicular dendritic cells of the lymphoid system, and the arachnoid and pia layers of the cerebral meninges [for review (5)]. In addition to desmocollin 3, the major protein components of desmosomes are desmoplakins 1 and 2, plakoglobin, plakophilin, desmogleins 1, 2, and 3, and desmocollins 1 and 2 (5). Previous immunohistochemical studies have demonstrated that reduced expression of desmosomal proteins is correlated with invasiveness and poor differentiation status in various types of human tumors (6-9). No reduction in expression of desmosomal components was observed in a study of colorectal cancers, however (10). In breast tumors, staining for desmoplakins 1 and 2 has been shown to be significantly reduced in moderately or poorly differentiated tumors as compared to well-differentiated tumors and normal breast tissue (11), and

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Abbreviations: 3' UTR, 3' untranslated region; EGF, epidermal growth factor; EST, expressed sequence tag; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HMEC, human mammary epithelial cell(s); PCR, polymerase chain reaction; SDS, sodium docedyl sulfate

Key words: down-regulation, desmocollin 3, breast cancer, desmosome, gene expression

levels of plakoglobin are decreased in invasive breast tumor cell lines as compared to weakly invasive MCF-7 cells (12). However, to date, there have been no reports of reduced expression of desmocollins in breast cancer. Here we present experiments which indicate the consistent down-regulation of desmocollin 3 in human breast tumors.

Materials and methods

Cell culture and reagents. Normal human mammary epithelial cells were obtained from two different sources: HMEC 1001-8 and HMEC 4144-2 cells were purchased from Clonetics (Walkersville, MD), and HMEC 184 cells were a gift of Dr Martha Stampfer (Lawrence Berkeley National Laboratory, Berkeley, CA). Also received from Dr Stampfer were HMEC 184B5 cells, a variant of HMEC 184 cells which have been immortalized by treatment with benzo(a)pyrene (13) but remain non-malignant (14). The human breast tumor cell lines BT-549, MCF-7, MDA-MB-231, MDA-MB-157, MDA-MB-134, SK-BR-3, T47-D, and ZR-75-1 were purchased from ATCC (Manassas, VA). HMEC 1001-8, HMEC 4144-2 and HMEC 184 cells were grown in MEGM media (Clonetics), and HMEC 184B5 and all tumor cell lines were grown in DMEM/10% FBS (Life Technolgies, Grand Island, NY) supplemented with 5 ng/ml human EGF (Upstate Biotechnology, Lake Placid, NY).

Primary human breast tumors. Samples of invasive ductal carcinomas of the breast (not otherwise specified), stage 1-2, were obtained from The Johns Hopkins Breast Tumor Bank (Johns Hopkins Hospital, Baltimore, MD). Tissues were obtained immediately after surgical resection and stored at -80°C until extraction of total RNA or protein. Microscopic examination of representative tissue sections indicated that samples contained greater than 50% tumor cells.

RNA extraction and cDNA synthesis. For Northern blots and TaqMan assays, RNA was extracted from cells cultured in 150-mm tissue culture dishes (Becton Dickinson Labware, Franklin Lakes, NJ) at 30-70% confluence using an RNeasy Midi Kit (Qiagen, Valencia, CA). RNA was further purified by extraction with TRIzol LS Reagent (Life Technologies). For Northern blots, poly (A^+) RNA was isolated from total RNA extracts using an Oligotex mRNA Mini Kit (Qiagen). RNA from primary tumors was extracted for TaqMan assays using only TRIzol Reagent (Life Technologies). cDNA was synthesized from 0.9 µg of each preparation of total RNA using Superscript II RNase H- Reverse Transcriptase (Life Technologies).

Isolation of 4.1 kb cDNA clone containing the sequence of I.M.A.G.E. clone 133534. A human placenta cDNA library from Origene Technologies (Rockville, MD) was screened by PCR analysis for the longest clone containing a sequence matching that of I.M.A.G.E. clone 133534, which was found to be down-regulated by cDNA microarray analysis (information on the I.M.AG.E. consortium's collection of cDNA clones is available at www.info@image.llnl.gov/). The library contains approximately 500,000 clones arrayed into 96 separate 96-well subplates. PCR screening of 96-well plates, and subsequently of individual colonies plated out on LB/agar/ampicillin plates, was conducted in 2 steps. In the first step, positive wells or colonies were identified using 2 gene-specific primers: 5'-CACCCACTGTGTTTTGCTCAC TCCCTC-3' (forward) and 5'-GCAAAACAAACCCCACTG CATTTTAGAC-3' (reverse), which bracket a 440-bp segment of I.M.A.G.E. clone 133534. In the second step, the size of the cDNA insert of positive samples was determined by PCR using the reverse gene-specific primer listed above along with a primer matching a T7 promoter sequence contained in the vector near the cDNA insert. PCR reactions were performed using Advantage cDNA Polymerase Mix (Clontech Laboratories, Palo Alto, CA). The one positive colony found was sequenced using Big Dye chemistry (PE Applied Biosystems, Foster City, CA).

Northern blot analysis. Two or 4 µg of poly (A^+) RNA from HMEC 1001-8 cells or human tumor cell lines was electrophoretically separated using a Northern Max-Gly Kit (Ambion, Austin, TX) according to the manufacturer's protocol, in which RNA is denatured in a load dye containing glyoxal and dimethylsulfoxide and separated on a 1% agarose gel containing no denaturant. A ladder of RNA size markers ranging from 0.28 to 6.58 kb (Promega, Madison, WI) was included on the gel. RNA was transferred onto a BrighStar-Plus positively-charged nylon membrane (Ambion) using a Transphor electro-transfer unit (Hoefer Pharmacia Biotech, San Francisco, CA) and crosslinked to the membrane by exposure to ultraviolet light in a Stratalinker (Stratagene, La Jolla, CA). Four riboprobes labeled with [α-^{32}P]UTP (NEN Life Science Products, Boston, MA) and designated as Dsc3 235, Dsc3 2900, GK01, and actin 326 were generated using a Strip-EZ RNA Kit (Ambion), according to the manufacturer's instructions. Briefly, primer pairs were synthesized for PCR amplification of 3 different regions of the desmocollin 3 cDNA and one region of the human ß-actin cDNA. A promoter sequence for the T7 RNA polymerase having the sequence 5'-TAATACGACTCACTATAGGGAGA-3' was added to the 5' end of the reverse primer of each pair to allow for the subsequent synthesis of riboprobe transcripts of the PCR products. The primers used were as follows: for the Dsc3 235 riboprobe, 5'-AGAGTTCTAAATGATGGGTCAGTGTA-3' (forward) and 5'-(T7 promoter)-CGATACCTTCTTCTGA TGTTCTAGCA-3' (reverse); for Dsc3 2900, 5'-CAGACAA CTGGTAAATCTCAAACTCCAGCACTG-3' (forward) and 5'-(T7 promoter)-GCTACTTCATAGGGCACTTCAAC ACATTTTGC-3' (reverse); for GK01, 5'-ACACCCACTGT GTTTTGCTCACTCCCTC-3' (forward) and 5'-(T7 promoter)-GCAAAACAAACCCCACTGCATTTTAGAC-3' (reverse); for actin 236, 5'-ATCTGGCACCACACCTTCT ACAATGAGCTGCG-3' and 5'-(T7 promoter)-CGTCA TACTCCTGCTTGCTGATCCACATCTGC-3' (reverse). The DNA template used in the PCR reactions with these primers was cDNA prepared from HMEC 4144-2 cells. Unincorporated nucleotides were removed from the products of the transcription reactions using Micro Bio-Spin 6 Chromatography Columns (Bio-Rad Laboratories, Hercules, CA). Each riboprobe was hybridized with a separate section of the blot overnight at 68°C in ULTRAhyb (Ambion), followed by washing 2x5 min at 68°C with both a low stringency and a

high stringency wash solution (4 washes total), both of which are part of the Northern Max-Gly kit, and are equivalent to (2X SSC, 0.1% SDS) and (0.1X SSC, 0.1% SDS), respectively. Autoradiography of the filters was performed both at -80°C and at room temperature using Hyperfilm-ECL (Amersham, Buckinghamshire, UK).

Real-time quantitative PCR. Real-time quantitative PCR reactions for measurement of desmocollin 3 mRNA levels were carried out using the TaqMan protocol on an ABI PRISM 7700 Sequence Detection System (PE Applied Biosystems). All reaction components, except for cDNA samples, were also obtained from PE Applied Biosystems. Reactions were assembled in triplicate using cDNA template from various normal or tumor breast cell lines or tissues, TaqMan Universal Mastermix, a pair of PCR primers, and a probe which is labeled at the 5' end with the reporter dye 6-carboxy-fluorescein (6-FAM) and at the 3' end with the quencher dye carboxytetramethylrhodamine (TAMRA). The primers 5'-CCCAAGACTTTACTAGTGCCGATAA-3' (forward) and 5'-CAAAGAACAAAGAGATGGTTAGAGGTT-3' (reverse) were used at a final concentration of 450 nM, and the probe 5'-(6-FAM)-TTCTCAAAGAGCAACCAGTATCACTTCC CTGTT-(TAMRA)-3' at 200 nM. To standardize the amount of RNA in each sample, expression of GAPDH in each cDNA sample was also measured in triplicate using the primers and probe included in the GAPDH Control Reagents kit.

Western blot analysis. Protein extracts from cultured cells were obtained by scraping cells into sample buffer (2% SDS, 10% glycerol, 60 mM Tris, 0.005% Bromophenol Blue, 0.1 M dithiothreitol) and boiling for 5 min. Extracts of primary tumors were obtained by chopping tumors into fine pieces, sonicating for 20-30 sec and heating for 10 min at 70°C in sample buffer, and passing through both 26-gauge and 30-gauge needles. Samples were stored at either -20°C or -80°C until separated on 7.5% polyacrylamide gels and then transferred to nitrocellulose membranes. The membranes were incubated with Dsc3-U114 primary antibody, which is a mouse monoclonal anti-desmocollin 3 (Research Diagnostics, Flanders, NJ), diluted 1:10 in TBST buffer (20 mM Tris, 137 mM NaCl, pH 7.6 with 0.1% Tween 20) containing 5% non-fat dry milk for 1 h at room temperature. The membranes were then washed 5x10 min in TBST, followed by a 30-min room temperature incubation with secondary antibody, which is a horseradish peroxidase-linked goat anti-mouse (Kirkegaard & Perry Laboratories, Gaithersburg, MD), diluted 1:5000 in TBST containing 5% milk. Membranes were washed 5x10 min with TBST and the proteins were visualized by ECL (Amersham).

Results

Identification of the cDNA insert of I.M.A.G.E. clone 133534 as part of the 3' UTR of human desmocollin 3. In a microarray analysis comparing human breast cancers to the immortalized non-malignant breast epithelial cell line HMEC 184B5, the sequence of the cDNA insert of I.M.A.G.E. clone 133534 was found to be consistently down-regulated in breast tumors (2). The cDNA insert is specified by 2 overlapping ESTs, which are single-pass sequence reads from the 5' and 3' ends

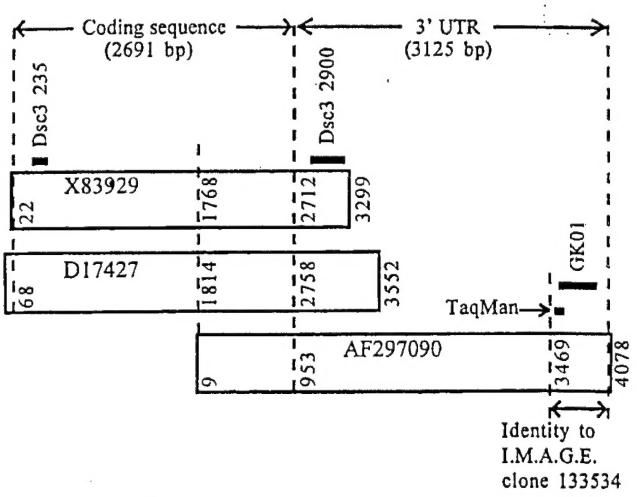

Figure 1. Alignment of 3 GenBank mRNA sequences for desmocollin 3. GenBank accession numbers X83929 and D17427 contain the complete coding sequence of desmocollin 3 but only the beginning of the 3' UTR, whereas AF297090 contains only part of the coding sequence but all of the 3' UTR. The nucleotide positions of the beginning and end of the coding sequence (including the stop codon), and the positions of regions which are complementary to riboprobes Dsc3 235, Dsc3 2900 and GK01 used in the Northern analysis presented in Fig. 2, are indicated for each accession number where applicable. Translation of an open reading frame at the 5' end of AF297070 is identical to 314 amino acids at the carboxy terminals of the 896 amino acid translations of X83929 and D17427. Also indicated is the region of AF297090 against which a probe and primers were designed for the TaqMan analysis presented in Table I. The riboprobe GK01 and the TaqMan primers and probe all hybridize within a region which has identity with the cDNA insert of I.M.A.G.E. clone 133534.

of the insert, indicating that the insert is approximately 600 bp in length. A search of GenBank using the BLAST sequence similarity search program yielded no matches, and so efforts were directed at isolating a longer cDNA clone containing this sequence. A human placenta cDNA library was screened by PCR, and a clone containing an insert of 4.1 kb was identified and sequenced. A BLAST search revealed that the 5' end of this longer clone has nearly perfect identity with more than 1,500 bp at the 3' ends of 2 mRNA sequences in GenBank for human desmocollin 3, which each contain the entire coding sequence for human desmocollin 3, but only part of the 3' UTR (GenBank accession numbers X83929 and D17427). The sequence of this clone has been submitted to GenBank and now has accession number AF297090. Because this cDNA comes from an oligo(dT)-primed library and contains a polyadenylation signal followed closely by a polyadenylation site, it is likely that it contains the extreme 3' terminus of the desmocollin 3 mRNA. The alignment of this cDNA sequence with the previously submitted sequences (Fig. 1) indicates that full-length mRNA for desmocollin 3 should be approximately 5.9 kb, which is in agreement with Northern blot analyses (3; also, see below). In support of this, we have obtained a 5.9 kb PCR product from a preparation of cDNA from 184B5 cells using primers directed against the 5' end of X83929 and the 3' end of AF297090, and sequencing of the ends of this PCR product verified the alignment shown in Fig. 1 (data not shown).

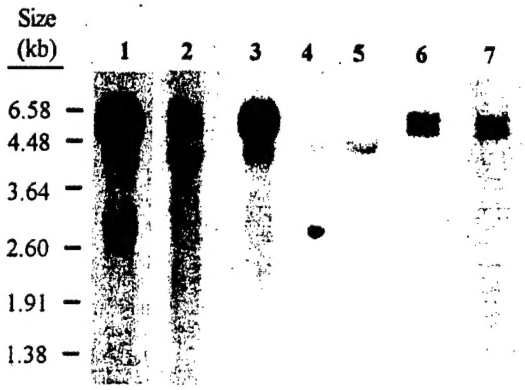

Figure 2. Northern blot analysis of Dsc3 expression in neoplastic and normal human mammary epithelial cell lines using three independent riboprobes. Samples containing 4 µg poly (A+) RNA from HMEC 1001-8 (lanes 1 and 2) or 2 µg poly (A+) RNA from either HMEC 1001-8, MCF-7 or BT-549 cells (lanes 3-5, respectively) were separated on a 1% agarose gel and electroblotted onto a positively-charged nylon membrane, as described in Materials and methods. The membrane was cut into 3 sections, and each section hybridized with one of three ^{32}P-labeled riboprobes. The Dsc3 235 probe (lane 1) is complementary to 147 bases near the 5' end of the Dsc3 coding sequence, whereas the Dsc3 2900 probe (lane 2) is complementary to 352 bases near the beginning of the 3' UTR, and the GK01 probe (lanes 3-5) to 440 bases near the 3' end of the 3' UTR (see Fig. 1). A film exposure at -80°C of 50 h was used for lane 1, 118 h for lane 2, and 30 h for lanes 3-5. To reveal that the Dsc3 bands of lanes 1 and 3 are each actually comprised of 2 bands, 12-h and 9-h film exposures of the samples in these lanes are shown in lanes 6 and 7, respectively. Stripping of the blot and rehybridization with a riboprobe complementary to the cDNA for human ß-actin verified that the amounts of RNA loaded into lanes 3-5 were approximately equal, as were the amounts loaded into lanes 1 and 2 (data not shown).

Northern blot analysis of desmocollin 3 expression in neoplastic and normal human mammary epithelial cell lines using three independent riboprobes. Northern blot analysis of poly (A+) RNA from HMEC 1001-8 cells, as well as the breast tumor cell lines MCF-7 and BT-549, are presented in Fig. 2 (see Fig. 1 for hybridization sites of probes). For HMEC cells, a band with a size of approximately 5.9 kb was observed with riboprobes directed against either the beginning of the coding region (lane 1), the beginning of the 3' UTR (lane 2) or the end of the 3' UTR (lane 3), further supporting the identification of GenBank accession number AF297090 as part of the desmocollin 3 mRNA sequence. The bands in lanes 1 and 3 are shown to actually consist of 2 bands in the shorter film exposures of these lanes presented in lanes 6 and 7, respectively. The difference in size of alternative splice variants previously reported for the desmocollin 3 mRNA is only 43 bp (3), which is too small to be resolved in Northern analysis. Thus, one of the bands of the doublet may be a previously unrecognized splice variant. When poly (A+) RNA from MCF-7 and BT-549 cells (lanes 4 and 5) is exposed to the same probe used for HMEC cells in lane 3, no 5.9 kb band(s) is observed, which indicates down-regulation of the mRNA for desmocollin 3 in these cells.

Real-time quantitative PCR analysis of desmocollin 3 expression. To better quantitate the degree of down-regulation of desmocollin 3 message in breast tumors, real-time quantitative PCR analysis using the TaqMan method (Perkin Elmer) was performed. A TaqMan probe and primer pair were designed against a region near the 3' end of the desmocollin 3 mRNA, as shown in Fig. 1. Desmocollin 3 expression in 6 breast tumor cell lines, 2 HMEC cell strains and 6 primary breast tumor samples were compared to that in immortalized, non-malignant HMEC 184B5 breast epithelial cells (Table I). Expression was down-regulated by a factor of between 90 and

Table I. Total RNA was prepared from cell lines or primary breast tumors and expression of Dsc3 mRNA was quantitated by a TaqMan real-time quantitative PCR assay, using GAPDH as an endogenous control to normalize for mRNA content.

Source of RNA	Dsc3 C_T (mean ± SD)	GAPDH C_T (mean ± SD)	Dsc3 down-regulation factor[a] (mean)
184B5	22.6±0.0	17.2±0.1	1.0
BT-549	31.3±0.2	19.4 ± 0.1	90.5
MCF-7	37.0±0.1	17.0±0.1	24,833.0
MDA-MB-134	34.1±0.4	17.3±0.0	2,702.4
MDA-MB-157	34.6±1.0	18.3±0.0	1,910.0
MDA-MB-231	35.8±0.3	18.1±0.1	5,042.0
T47-D	36.7±0.7	18.9±0.3	5,792.0
HMEC 4144-2	24.0±0.2	17.8±0.2	1.7
HMEC 1001-8	25.1±0.3	18.7±0.3	2.0
Human primary breast tumor 1	27.0±0.2	18.0±0.2	12.1
Human primary breast tumor 2	27.0±0.1	18.3±0.1	9.8
Human primary breast tumor 3	31.6±0.3	21.3±0.1	29.9
Human primary breast tumor 5	29.6±0.1	23.7±0.3	1.4
Human primary breast tumor 6	33.0±0.2	23.5±0.1	17.1

The C_T value indicates the cycle at which the fluorescence resulting from generation of PCR product rises above background. The Dsc3 down-regulation factor expresses the fold-reduction in Dsc3 expression relative to expression in 184B5 cells, and is calculated by the $\Delta\Delta C_T$ method, using the below formulae. [a]In a given sample, Dsc3 down-regulation factor = $2^{(\Delta\Delta C_T)}$, where ($\Delta\Delta C_T$) = (ΔC_T for sample) - (ΔC_T for 184B5), and (ΔC_T for sample) = (Dsc3 C_T for sample) - (GAPDH C_T for sample).

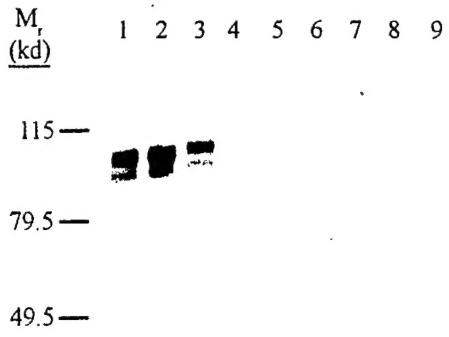

Figure 3. Western blot analysis of Dsc3 expression in normal and malignant human breast cell lines. Proteins were separated by SDS-PAGE, transferred to nitrocellulose membranes and incubated with the mouse monoclonal antibody Dsc3-U114, which reacts specifically with human desmocollin 3 (16). Approximately equal amounts of protein were loaded in all lanes, as verified by preliminary staining with *Ponceau S*. Both splice variants of desmocollin 3 were detected in protein extracts from non-malignant human breast cell strains 184B5 at passage 40 (lane 1), 184B5 at passage 126 (lane 2) and 184 at passage 13 (lane 3), but not in extracts from breast tumor cell lines MCF-7 (lane 4), ZR-75-1 (lane 5), MDA-MB-231 (lane 6), MDA-MB-157 (lane 7), T47-D (lane 8), and SK-BR-3 (lane 9).

Figure 4. Western blot analysis of Dsc3 expression in primary human breast tumors. As in Fig. 2, separated proteins were incubated with monoclonal antibody Dsc3-U114. Subsequent staining of the blot with a monoclonal antibody against human ß-actin verified that approximately equal amounts of protein were loaded in all lanes (data not shown). Positive staining for desmocollin 3 was obtained with 184 cells at passage 13 (lane 1) and 184B5 cells at passage 40 (lane 2), but not MCF-7 cells (lane 3), or the primary human breast tumors 7136 (lane 4), 7121 (lane 5), 7103 (lane 6), 7235 (lane 7), 7130 (lane 8), 7121 (lane 9), and 7111 (lane 10). Staining for desmocollin 3 in MCF-7 and all of the primary tumors could not be detected even with a 10-fold increase in the duration of the film exposure used to detect antibody binding (data not shown).

24,000 in breast tumor cell lines, and by a factor of 10-30 in primary tumors, with the exception of one primary tumor in which expression was not significantly reduced. In that sample, the presence of a large number of non-tumor cells might possibly explain the failure to observe down-regulation. Expression in the normal breast epithelial cell strains HMEC 4144-2 and HMEC 1001-8 was comparable to that in HMEC 184B5 cells. The high level of expression of desmocollin 3 in human mammary epithelia observed here is in agreement with a previous study demonstrating abundant immunohistochemical staining for desmocollin 3 in bovine mammary gland (15).

Western blot analysis of desmocollin 3 expression in breast tumor cell lines and primary breast tumors. To determine whether down-regulation of desmocollin 3 also occurs at the protein level, Western blot analysis was performed using a monoclonal antibody which is specific for desmocollin 3 (16). The expression of desmocollin 3 protein in immortalized HMEC 184B5 cells and the non-immortalized parent cell strain HMEC 184 is compared to that in 6 breast tumor cell lines in Fig. 3. Two bands with molecular weights in the 100-110 kDa range, corresponding to the 2 splice variants of desmocollin 3 (3,16), are present in extracts of HMEC 184B5 cells at passage 40 (lane 1) and at passage 126 (lane 2), and in HMEC 184 cells (lane 3). Bands are not detectable in any of the tumor cell lines, even when autoradiographic film exposure times are increased several-fold. Since desmocollin 3 is expressed in both normal HMEC and immortalized HMEC, at both an early passage and late passage, but not in any of the breast tumor cell lines, down-regulation of desmocollin 3 expression appears to result not simply from immortalization or from prolonged maintenance of cells in culture, but rather from malignant transformation. The extension of these results to primary tumors is shown in the Western blot presented in Fig. 4, in which desmocollin 3 protein was detected in HMEC 184 cells (lane 1) and HMEC 184B5 cells at passage 40 (lane 2) but not in any of the primary breast tumor samples.

Discussion

Previous studies have presented evidence suggesting that a reduction in desmosome-mediated adhesion to neighboring cells may contribute to the invasive behavior of tumors (6-12). Of the studies involving breast cancer, only desmoplakin and plakoglobin have been reported to have exhibited altered expression (11,12). Desmocollin 3 expression has been addressed only indirectly. An extensive immunohistochemical study of oral squamous cell carcinomas (7) demonstrated that the combined expression of desmocollins 1, 2 and 3 (as well as desmogleins and desmoplakins) is inversely correlated with the presence of lymph node metastases, and that expression of these components is significantly reduced in poorly differentiated as compared to well differentiated tumors. An antibody which recognizes all three isoforms of desmocollin was utilized in that study, and therefore expression of desmocollin 3 was not specifically assessed. We have presented the first report of the down-regulation of desmocollin 3 in any human tumor type.

Whether reduced expression of one or more desmosomal components in protein extracts prepared from breast tumor cell lines or primary breast tumor samples is indicative of a reduction in either the number of desmosomes or their adhesive function remains to be established. Electron microscopy studies will probably be needed to answer this question. A reduction in the number or functionality of desmosomes would likely result in reduced adhesiveness to neighboring cells, which might in turn lead to increased invasive and metastatic potential. In support of this contention, it has been demonstrated that the invasiveness of non-adhesive L929 fibroblasts *in vitro*, as measured by collagen invasion assays,

can be reduced by transfection of the cells with a combination of desmosomal components which enhances their adhesiveness (17). Also, an electron microscopy study of transitional cell carcinoma of the human urinary bladder found a correlation between numbers of desmosomes and aggressiveness (18), and similar findings have been reported for carcinoma of the cervix (19). Thus, desmosomes appear to play a role in suppressing metastasis. Moreover, there is increasing evidence suggesting that desmosomes are involved in signal transduction (5), and therefore may potentially exert an influence on signaling pathways controlling cell proliferation. If so, disruption of desmosome-mediated signals might contribute to the neoplastic transformation of epithelia.

Certain desmosomal components may be more important than others in suppressing either the development or invasiveness of tumors. Desmocollin 2, for example, has not displayed consistent up- or down-regulation in cDNA microarray studies of human breast tumors (2,20). The consistent down-regulation of desmocollin 3 in breast tumors suggests that it may serve a particularly important role in the suppression of metastasis and or tumorigenesis, and indicates the need for further studies.

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